Further Studies on Restitution-bodies and free Tissue-culture in Sycon.

By

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With Plates 13 and 14.

Contents.

1. Introduction.

In a previous paper (Huxley, **7**) I showed that the remarkable phenomena of regeneration from dissociated cells, first observed by H. V. Wilson (**15**) in monaxonid sponges, later extended by him (**16**) and by de Morgan and Drew (**4**) to coelenterates, could be studied in a simpler and more satisfactory form in

heterocoelous calcareous sponges than in any other types investigated. I further showed that, by certain methods, restitution-bodies composed entirely or almost entirely of collar-cells could be produced, and that these assumed a form quite unlike anything found in normal sponges, but with a resemblance to a Choanoflagellate colony. Simple excess of collar-cells, or, apparently, larger masses composed almost entirely of collar-cells, led to the formation of what I called choanocyte blow-outs—a part of the solid mass becoming blown out to form the segment of a collar-cell sphere.

Since then I have continued making observations on the subject as opportunity offered. Although these cannot pretend to completeness, they have brought certain new facts to light, which I publish in the hope that other workers may extend them by observation on the same favourable material. Some of the work was done at Wood's Hole, Massachusetts, and some at the M.B.A. Laboratory, Plymouth. I have to thank the authorities at both institutions for their help in getting material and in other ways. I have also to acknowledge much efficient help at Wood's Hole from Mr. I. J. Davies, laboratory assistant in the Rice Institute, Houston, Texas.

2. Material and Methods.

A species of Sycon was used at both places. That used at Plymouth was S. coronatum, obtained from piles in the Millbay docks. Orton (12) has recently drawn attention to the fact that this sponge grows actively during the winter without reproducing; but during the summer it reproduces so long as the temperature is above a certain level, and scarcely grows at all. The same is to be presumed true of other species of the same genus. If so, it follows that the best time for conducting similar experiments will be during the cooler half of the year.

Experiments were tried on homocoelous sponges such as Clathrina and Leucosolenia, but without much success. Restitution masses are formed, but are small and do not live well. The collars and flagella are withdrawn on very slight provoca-

tion, and the organisms and their parts appear to be more delicate.

The method originally adopted was that discovered by H. V. Wilson—the squeezing of the chopped-up sponge through fine-meshed silk bolting-cloth.

In order to procure ' pure cultures ' of collar-cells, the sponge or a transverse segment of it is held with one needle and briskly teased with another. By this means large sheets of collar-cells are obtained. If the pieces are shaken together in a solid watch-glass, they will cohere and larger masses result.

A method which will give an excess of collar-cells but not an almost pure culture is simply to perform the teasing process as above, and then remove the portions of original sponge. The collar-cells, being more easily detached than the others, will form the bulk of the tissue present.

Finally, simple squeezing of the whole sponge with the fingers into water will give a thick suspension of single cells and very small cell-aggregates, which is very similar to the culture produced by squeezing through gauze. By different dilutions of this suspension, different results can be achieved.

These methods will be called squeezing through gauze, choanocyte isolation, teasing, and squeezing without gauze respectively.

The experiments at Wood's Hole were done in late July and August ; those at Plymouth in July and early August.

3. Subdivision of Restitution-bodies.

(Work done at Plymouth.)

A teased culture was made on August 3, 1920. Many of the restitution masses were of rather large size. They began to blow out in normal fashion, and after six days a number of very fine choanocyte blow-outs were present. On the seventh day they were even better. On the eighth day a certain quantity of bodies consisting of a number of small spherules, rather closely packed together, were observed in the dish (fig. 1). They were attached to the glass, and some force was

necessary to squirt them free. On examining them I at first thought that they might be derived from the Sycon restitution-bodies, but dismissed the idea as improbable. Later in the same day I took some of them to Miss Lebour, Naturalist at the Laboratory, to see if she could identify them. On examining them under pressure with a high power, it was found that they contained fragments of spicules. Thus the suspicion that they were of sponge origin was strengthened.

Two days later a restitution-body which had for four days been isolated for other purposes on a slide in a moist chamber was examined and was found to have subdivided into six spherules (fig. 2, *a*). Thus their sponge origin was conclusively proved. Meanwhile the original dish was picked over, some of its contents preserved, and the remainder separated into divided and normal undivided masses. The normal masses were examined two days later (the tenth day of the whole experiment) and found to be still undivided, many with active flagella and protruded collars still visible externally. On the thirteenth day, eight out of fourteen masses were still single, but the remaining six had subdivided. They were similar in every way to those observed on the eighth day, except that they were not so closely packed, and that I could see no traces of a gelatinous membrane round the spherules. It would, however, of course be expected that those which subdivided earlier would be of slightly different composition from these later-divided ones.

A detailed observation of one of the earlier divided masses on the ninth day (fig. 1, *a*) showed that the spherules were tightly packed and mutually compressed. The whole body was surrounded by a faint gelatinous membrane, which apparently caused the whole to adhere to the glass. Under a higher power (fig. 1, *b*) the single spherules were seen to consist of a one-layered epithelium surrounding a central mass. The epithelium was composed of extremely clear cells, with a few minute granules; the central mass did not touch the epithelium at all points, and was dense and of a yellowish colour; cell outlines were not visible in it. The single spherules did not

appear to possess separate membranes. My attention, however, had not yet been drawn to this point, and I cannot be sure of it. Broken spicules were present in some. Another mass examined on the same day contained many more fragments of spicules. It was very similar to the first, but the epithelia were not so sharply marked off from the central masses, which in their turn were not quite so dense. When gelatinous membranes were present, numerous bacteria were usually seen along their outer edges.

The spherules were of various sizes, as is shown in fig. 4, which illustrates an isolated specimen on the tenth day. This same specimen was examined again on the thirteenth day. The same individual spherules were identified, but their appearance had changed, their outlines being less regular and the general effect more transparent. On examination with a high power this was seen to be due to the fact that in the majority most of the individual cells had separated from each other (fig. 5). Each spherule was surrounded by a definite layer of jelly. Within this, isolated clear cells, all sub-spherical, were scattered. At one point, either central or at the side, a denser yellowish mass was seen. This appeared to consist of larger cells, still adherent, containing many granules (of two types, large and small). A few of the small clear cells could still be seen embedded in some of the yellow masses. A minority of the spherules showed a different appearance (fig. 6). In them the spherule had simply subdivided into a small number of pieces, of somewhat irregular shape, each apparently consisting of clear cells round the periphery, yellow cells within. Finally, one or two spherules intermediate in type were seen, i. e. with a few large masses and also some isolated clear cells.

The independent gelatinous coverings of the separate spherules were also seen in other specimens, e. g. in that shown in fig. 2.

A variant of the types already discussed is shown in fig. 3, which illustrates a small mass found in the culture-dish, consisting of an epithelium of dermal cells surrounding a central mass, presumably mainly of collar-cells, which had subdivided

into spherules. No gelatinous layer was seen round this mass. This was paralleled in the development of some other masses ; e. g. that shown in fig. 2, *a*, had, three days later, assumed the appearance shown in fig. 2, *b*. The smallest spherule was unchanged. The remaining five, however, were all surrounded by a well-marked epithelium of dermal cells very different from the epithelium shown in fig. 1, which I take to be choanocytic. The masses had swollen up by the secretion of fluid. The central portion had in three of the spherules begun to fragment. The gelatinous layers were of the same thickness as before. Although no cell-outlines had been visible in the spherules when examined three days previously, it had been noticeable that their outer boundary was very sharp. Other observations give colour to the idea that this sharp boundary heralds the formation of a dermal epithelium.

In this case the dermal epithelium is formed after the spherules have been produced. In the mass shown in fig. 3, the whole mass forms a dermal epithelium, and the spherules are then produced internally.

The further history of the spherules was as follows. The majority showed disintegration of the types shown in figs. 5 and 6. A few degenerated directly. No recovery was observed in spite of change of water. This, however, may only mean that laboratory conditions were unfavourable. It is to be remarked that the general appearance of the tissues in stages like that of fig. 5 was perfectly healthy.

Another culture was later found, where the same processes were observed. It was unfortunately not possible to carry out experiments to determine if subdivision could be initiated at will.

The subdivision appears to be primarily a reaction to unfavourable conditions (witness the accumulation of bacteria round the edges of the subdivided masses). In all the dozens of dissociation cultures I have made at Naples, Wood's Hole, and Plymouth, these two were the only ones where subdivision was observed. Both these cultures were from teased, not squeezed, material.

The secretion of gelatinous membranes is of interest. Other noteworthy points are as follows :—(1) The size of the spherules produced varies within considerable limits. Those produced by a single mass might be approximately equal, or of very different sizes. (2) I at first thought that the phenomenon was determined by the proportion of dermal cells present, subdivision continuing until enough surface was formed to accommodate all the dermal cells in the state of simple epithelium. Appearances like that of fig. 1, *b*, however, seem to negative this, for there the epithelium surrounding the spherules is cuboidal, and quite unlike any dermal cells seen by me. This epithelium seems to consist of the healthiest choanocytes present. The difference of colour between them and the cells of the inner masses, however, is to be noted, and it is p o s s i b l e that they represent a dedifferentiated condition of the dermal cells. If so, they would resemble the cuboidal form of the ectoderm cells seen in dedifferentiated stolons, &c., of the Ascidians, Perophora and Clavellina-cells which are normally as flattened as the normal dermal cells of Sycon. At all events the phenomenon must be determined by some surface-volume relation, the cells not being able to cohere in large masses when in certain conditions.

In any case, the spontaneous segmentation of the masses into regularly-arranged portions of smaller size is of interest. This phenomenon never occurs, as far as is known, in the normal life-history of Sycon ; yet the process is regular, and at first sight would be taken for a normal occurrence. It is an example of the determination of physiological processes by the direct action of external circumstances, without any modification by way of heredity. A somewhat similar phenomenon was found by Müller (**10**) in restitution-bodies of Spongilla, but it was not so regular, nor, since it only occurred in large masses, does it seem to have been due to identical causes.

(3) The separation of the clearer cells from each other, apparently when the circumstances have become slightly more unfavourable, is also of interest. In Perophora and in Hydroids, a slight concentration of toxic substances starts dedifferentiation

in the zooids (results in course of publication). The further progress of events in these organisms, however, is determined by the emergence of the cells from the tissue into the blood, leading to the resorption of the zooid. Here, in these spherules, the cells emerge from the tissues, but must remain in position, since there is no means by which they can migrate elsewhere. Slight mercury poisoning also causes emergence of some of the endoderm cells from the gut of late Echinoid plutei. It is probable that total or partial resolution of the tissues into separate cells is a general occurrence in dedifferentiation, but that it is masked in many cases, e. g. in Clavellina. A study of these phenomena, together with that of dissociation of cells as observed in particular chemical solutions, as. e. g., observed by Gray (**5**), will throw light on the problem of cell-coherence in general.

(4) The production of a definite dermal epithelium late in the history of many subdivided spheres is to be considered in relation to the observed fact that restitution-bodies with dermal epithelium are more viable than those composed of choanocytes alone (Huxley, **8**).

(5) The transition from a state in which no cell-outlines are visible (figs. 2, *a* ; 6) to one where the cells are distinct (fig. 2, *b*) or separated (fig. 5) is to be compared with the formation of syncytia in Coelenterate restitution masses, as noted by Wilson and by de Morgan and Drew, and their subsequent resolution into cells. Here again a very important general phenomenon is made accessible to study.

.4. Dermal Blow-outs.

In my previous paper three types of restitution were described, leading to : (1) normal regenerates, consisting of dermal and gastral cells in normal proportion. These formed spicules, and those that lived long enough produced normal miniature sponges. (2) Collar-cell spheres : small hollow spheres. consisting of a single layer of choanocytes, with no, or very few, other cells. (3) Collar-cell blow-outs : masses consisting mainly of collar-cells, blown out in one or more regions

to form segments of spheres. The external epithelium of the solid remainder might be formed: (*a*) by collar-cells only, (*b*) by dermal cells only, (*c*) by patches of both.

Other types have now been observed. The most interesting are the dermal blow-outs. These appear to be formed whenever the mass contains a preponderance of dermal cells. A mass of collar-cells generally fills most of the interior: it is covered closely with a single layer of dermal epithelium, which at one point is swollen out to form a segment of a sphere which thus consists entirely or almost entirely of dermal cells. Often cells are to be seen adhering to its inner surface; these were sometimes rounded and of a fair size, presumably typical amoebocytes, oftener of the minute elongated type which I have called finger-cells (see p. 304). A few detached cells might sometimes occur in the cavity. These were occasionally seen to be forming spicules. A typical mass of this kind is shown in fig. 12.

Shaking caused contraction and disappearance of the blown-out regions, as with the collar-cell blow-outs.

One very peculiar mass was seen (at Wood's Hole). This was isolated together with a number of others shortly after concrescence, when they were solid and irregular in shape. Four days later this was found to have a large hemispherical collar-cell blow-out, which in its turn showed a small dermal blow-out on one side. Under the surface finger-cells were visible. It would thus appear that local as well as general excess of dermal cells can occur, leading to the formation of mixed blow-outs.

Previous experience (Huxley, **8**) has led me to conclude that when a small proportion of dermal cells is present in a culture, they exercise an attraction for each other. This leads to the production of a few normal regenerates in a culture consisting mainly of collar-cell blow-outs. In a similar way this congregation of dermal cells can lead to the formation of dermal blow-outs. This was so in the mass shown in fig. 12.

This and other facts would indicate that the formation of dermal blow-outs is mainly a matter of the number of dermal

cells present in a particular mass. That this is not all, however, is shown by the following experiment (Wood's Hole).

July 12. Several sponges squeezed through bolting-silk into a finger-bowl. Three dilutions of the resulting cell-suspension were made: (1) dense, (2) medium, (3) dilute.

Results:—After one day: (1) large, sometimes irregular, masses; (2) medium-sized spheroids, many with good collars and flagella, many with larvae embedded in them; (3) as (2), but smaller, and fewer with larvae.

After two days: (1) no blow-outs; most seem normal restitution masses; (2) most with collar-cell blow-outs; (3) some with collar-cell blow-outs.

After three days: (1) as before: (2) fewer collar-cells than the previous day; (3) none seen blown out.

After five days: (1) the smaller masses forming small dermal blow-outs; (2) many with large dermal blow-outs; (3) solid.

After nine days: (1) mostly dead; (2) many attached to glass; (3) as before, none attached.

A repetition of the experiment gave similar results, except that dermal as well as choanocyte blow-outs were formed early in the middle dilution.

It will thus be seen that dermal blow-outs did not begin to appear until the fifth (or fourth) day, and that they appeared most notably in the same culture which had previously produced the best choanocyte blow-outs. Their failure to appear in the large masses of (1) may be due to the fact that these in this experiment were not very healthy. It would appear, since the only difference between (2) and (3) lay in the size of the masses formed, that the eventual production of dermal blow-outs is determined partly by the total, and not only by the relative number of dermal cells present. It appears that first of all the collar-cells on the surface protrude collars and flagella towards the water; these are, however, very susceptible to noxious influences, and as culture conditions became less good they retracted into a spheroidal form. The dermal cells then migrated to the surface and covered the collar-cells with an epithelium, which they were apparently unable to do when

the external collar-cells were functional. Since, however, the total number of dermal cells in a mass is proportional to its volume, while the number required to form a single external layer of epithelium is proportional to its surface, there will be in large masses an excess of dermal cells above those needed to form the epithelium. This excess apparently forms the dermal blow-outs. The replacement of choanocytes by dermal covering is of interest in view of the greater viability or protective capacity of the dermal cells shown by other considerations (Huxley, 8).

Fig. 13 shows another type. A number of very large masses had formed in a culture produced by squeezing without gauze. The larger masses had first been very irregular in shape, and had demonstrably been formed by the coherence of original smaller spheroids. (The culture was made in a finger-bowl. The flat bottom was covered with small spheroids, while a ring of the large irregular masses was found at the foot of the sides. This was due to the opportunity given here for many masses to come in contact by rolling down the steep sides.)

These irregular large masses later rounded up, and shortly after this produced blow-outs. Some were similar to that seen in fig. 12. Others, however, consisted of a much-distended sphere surrounded by an epithelium of dermal cells, the contained gastral cells not forming a well-marked mass, but spread in layers of varying thickness over part of the inner surface of the sphere (fig. 13). The majority of the larger masses in the culture were of this type, while the majority of the smallest were not blown out at all, but were normal regenerates. This bears out the conclusion drawn above as to the rôle of size of mass.

Wilson, in his experiments with Coelenterates, also found that size of mass was very important, the larger masses failing to metamorphose. A study of restitution-bodies from this point of view would probably throw light upon the reasons for the sizes of the larvae in many low types.

An interesting feature of most dermal blow-outs examined

with the high power was the association of the small types of amoebocytes I propose to call finger-cells with the dermal cells in the blown-out region. This was observed both at Wood's Hole and at Plymouth. In surface view the dermal epithelium is seen to consist of a number of granular areas (fig. 12)—cell-bodies—separated by transparent areas, where the protoplasm is extremely thin. Cell-junctions cannot be seen in vivo. Below each granular area is seen an irregular stellate figure. On careful examination this is seen to consist of a number of finger-cells radiating from below the centre of the cell-body. Optical section of the periphery gives a profile view, when the body of the dermal cell is seen to be sharply marked off from the underlying finger-cells. Similar finger-cells are seen to protrude, but singly, from the inner mass of choanocytes. The meaning of this arrangement of finger-cells remains obscure.

The cultures containing the large dermal blow-outs above referred to were examined again later. Almost all had produced some spicules, and a considerable proportion had metamorphosed into functional young sponges of the ' Olynthus ' stage.

In my previous work (Huxley, 7, p. 169) I never obtained fixed Olynthus stages from restitution masses. Here, however, some 25 per cent. of them were firmly attached. A few of these were of great regularity of form (fig. 7, *a*), again surpassing any obtained previously. Others, however, showed marked irregularities, more pronounced than any seen in 1910, often appearing as if preparing to form a second osculum. In no case, however, was a second osculum seen, or even a rudimentary second oscular crown of spicules. The most remarkable thing about these forms was the large size shown by many of them, far exceeding that of a newly-metamorphosed larva. Thus, although large size is associated with less viability of restitution masses, yet even very large masses, provided they remain healthy, can metamorphose into normal-type Olynthi if the various sorts of cells are present in correct proportions.

Some idea of the normal size of Olynthi from larvae can be got by comparing the figures of larvae (figs. 9, *a*; 10, *a*,

drawn to a larger scale than figs. 7, *a* and *b*). It is possible that the abnormal-shaped Olynthi were produced by dermal blow-outs of the types of fig. 12, or by coalesced masses of irregular shape.

Numerous other masses, however, were seen of the type shown in fig. 8. Here spicule formation had progressed well, but no osculum was present. The most noticeable point was the restriction of the gastral layer to part of the sphere (as already seen, e. g. in fig. 13). The gastral layer was usually one cell-layer in thickness, but in some masses was several layers thick at certain spots only (fig. 8).

The disproportionate number of dermal cells had certainly delayed development. As I had to leave Plymouth the day after discovering this type of regenerate, their fate could not be ascertained.

5. Dark-centred Masses.

These were both seen at Wood's Hole and at Plymouth. Typically (fig. 11), they consisted of a dermal epithelium, surrounding several layers of pale cells, apparently choanocytes, which in their turn surrounded a central mass of dark yellowish-brown material, whose cellular nature could not be seen in vivo. The central mass is separated from the pale cells by a space. In one or two cases the central body was seen to be revolving. If this was so, then the collar-cells must have developed flagella on their inner side.

The meaning of these masses is obscure. There is a strong resemblance between the inner mass and the yellow-brown masses seen in the subdivided spherules (fig. 5), and some resemblance also between the intermediate pale layer and the isolated cells of fig. 5.

A few specimens were observed where a dark central mass was present, together with active choanocyte epithelium on the outside.

6. Adherence of Larvae to Masses.

Both at Wood's Hole and at Plymouth it was noticed that when cultures were made from sponges containing nearly

mature larvae, these might adhere to and be actually embedded in the restitution-bodies (figs. 9, *a*; 10, *a*). In this situation their flagella would continue to beat. Transferring masses with embedded larvae by means of a pipette often resulted in detaching the larvae (fig. 10, *b*). Larvae that remained attached appeared to become resorbed into the masses, finally disappearing (fig. 9, *a–c*). Histological investigation of this has not yet been undertaken.

7. Adhesion and Unification of Restitution-bodies.

In section 4 an account has already been given of the fusion of a number of spheroidal bodies to form irregular masses, which later became spheroidal in their turn. These were all masses with excess of dermal cells. Some observations on masses with excess of choanocytes may also be given. Some four-day restitution-bodies were isolated in a hanging drop. The chief are shown in fig. 9, *a*. Most are covered with dermal cells, but two have dermal epithelium in part. Some have attached larvae. After two days these were seen to have fused (fig. 9, *b*). Three larvae and two other small restitution-bodies, not shown in fig. 9, *a*, had not shared in the fusion.

On the next day the larvae were still visible, but the general form was not so irregular. The day after (fig. 9, *c*) the larvae were no longer visible, the blown-out region had increased, and the traces of the separate original masses have been almost lost, the mass looking quite unitary, though with slight irregularities. Two days later (fig. 9, *d*) slight regressive changes had set in. The form was more unified, but the blow-out was smaller, and the collars had been entirely, the flagella partially, retracted. Gaps in the blow-out appeared, bridged by dermal cells. Three days later the blow-out, together with all traces of flagella, had disappeared, and four days later the mass had still further shrunk, and was apparently covered entirely with dermal cells, though I could not be quite sure of this.

The most interesting feature of this is the gradual assumption of unitary form by artificial aggregation of cell-masses, which

in their turn are produced in a totally abnormal, artificial way. The form produced, however, as also in the case of the choanocyte spheres, though typical, is not in the least like anything occurring in the normal life of the species. Here, typical form and form-changes of an organic type are seen in artificial aggregates. Once more we see a series of organic forms very clearly as an equilibrium between external environment and inner constitution. Here, however, the inner constitution is simple, the changes are not running in grooves of heredity. It is probable that many adult forms of simple organisms, as well as simple developmental forms, are in this way determined almost entirely by a direct relation of not highly-differentiated tissues with environment. The blastula, for instance, may or may not represent an ancestral adult form. It certainly is a primitive ancestral developmental form ; but it is this not for any adaptive or eventually ' organismal ' reason, but because it is the simplest way in which a number of undifferentiated cells can arrange themselves in a fluid medium. See also Child (2) for an account of the way in which adult form may be largely determined thus in flat-worms.

8. Restitution-bodies and Tissue-culture.

It is obvious that the spheres produced by isolation of sheets of collar-cells are ' tissue-cultures ' in that they consist of one sort of cells only. Their history in ordinary sea-water is a history of gradual starvation, followed by involution, since the fluid does not contain sufficient nutriment. Numerous experiments were tried with a view to finding a suitable nutrient medium, but so far without success.

(1) Pure culture of the Diatom Nitzchia, so successfully employed by Allen and others for feeding Echinoid plutei, were obtained and mixed with water containing preponderatingly choanocyte restitution-bodies. In a few cases, diatoms were seen in the collars of collar-cells, or partly embedded in the cell-body ; but they were apparently too long for convenient ingestion.

(2) Suspensions of common sea-water Bacteria killed by heating were added daily.

(3) Masses were put in vessels, together with fresh Ulva, to see whether they would ingest the swarm-spores.

(4) Sterile solutions of Peptone in sea-water, of various strength, were prepared. The restitution-bodies were transferred to this through four changes of sterilized sea-water, the pipette being sterilized between each operation. Although somewhat over 50 per cent. of the cultures thus prepared became contaminated, yet a number remained free of bacteria. In all these, however, the choanocytes underwent regressive changes, actually sooner than in normal sea-water, and the masses died within a few days.

(5) 'Sponge Broth'. 3 c.c. of chopped sponge was extracted in 20 c.c. of sea-water and sterilized, and restitution-bodies transferred to it as under (4), but again with no success.

(6) Ammonium lactate of 0·1 per cent. concentration was prepared and a trace of phosphate added (cf. Peters, **14**), and then sterilized. Again some restitution-bodies were transferred to the medium without infection, but all contracted and died speedily.

(7) Under gauze, in the circulation (at Wood's Hole). Unfortunately these experiments had to be discontinued. The restitution-bodies remained healthy for some time, but growth could not be detected. The fact that normal regenerates throve better and actually grew in these conditions, warrants the belief that some modification of this method might be successful.

Although all methods so far tested have proved unsuccessful, yet I feel sure that choanocyte masses could be supplied with food. It is possible that experiments in circulation would succeed best at Naples, where Sycon establishes itself and grows in the tanks. Further, experiments of this nature would most profitably be undertaken in the cooler months (see § 1 of this paper). At Wood's Hole I found that covered cultures kept cool in the circulation throve better than those exposed to air-temperature.

The cultivation of the collar-cell spheres, if successful, would open out many points of interest. What, e.g., would happen if considerable cell-multiplication took place? The resemblance of the collar-cell spheres to colonial protozoa, and the fact that the collar-cells are the nutritive organs of the sponge, make the research still more interesting. Finally, the ease with which sheets of pure collar-cells can be obtained, and the fact that they will remain healthy, with expanded collars and active flagella, for one to two weeks without being fed, renders them very suitable as material. Detached tissues which assume characteristic form in this way and live for a considerable period in the normal medium may be termed free tissue-cultures.

One or two interesting points concerning ingestion by the collar-cells may be mentioned here.

(1) Addition of powdered carmine to a culture of choanocyte masses was followed within an hour or so by ingestion of some of the particles. Very many particles adhere to the flagella, so that the masses appear reddish. Such particles as find their way within the collar are ingested by a pseudopodial extension of the intrachoanal protoplasm. No extrachoanal ingestion was observed.

(2) When Nitzchia was added, very few were ingested, and these only partially. They were usually caught, like the carmine particles, by the ends of the flagella, and lashed to and fro. This adhesive condition of the flagella is of interest. (In fresh dissociation cultures, finger-cells may often be seen adherent to the flagella and being waved from side to side with their beat.) Some were also seen adherent to the inner side of the collars.

9. Mechanical Shock. Toxic Agents.

Mechanical shock, such as repeated pipetting, or even transfer to a hanging drop, will cause marked changes in the cells, both dermal and choanocytic. A collar-cell blow-out treated in this way shows marked reduction of the size of the blow-out region.

together with a thickening of its walls. The collars are usually retracted. (See figs. 10, *a*, and 10, *b*.)

This sensitiveness to mechanical stimuli is shown by many other tissues in culture (cf. Holmes, **6**).

Exposure to very dilute solution of mercuric chloride in sea-water $\left(\frac{n}{500,000}\right.$ to $\left.\frac{n}{2,000,000}\right)$ causes retraction, first of the collar and then, gradually, of the flagellum, together with slowing of the flagellar beat. The effect is proportional to the strength of the solution. This retraction of the flagellum is a remarkable phenomenon, and the short stumps of the flagella still beating provide a curious spectacle.

A record of an experiment is appended.

Record of Experiment.

Five or six collar-cell blow-outs in each solution (100 c.c. each).

A. Control. Collars and flagella remained normal for twenty-four hours.

B. $\frac{n}{1,500,000}\ HgCl_2$.

1 hr. 25 m. Two masses with short collars. Three masses with very short or no collars, and sharp smooth outline of epithelium, all with fair to good flagellar movement.

2 hrs. 10 m. None with more than very short collars. Some with short flagella. These beating faster than unretracted flagella of other masses. Smooth outline still visible.

19 hrs. No collars. Only two with flagella (moderate length), two with slight cell-disintegration.

C. $\frac{n}{750,000}\ HgCl_2$.

1 hr. 10 m. One mass with short collars in most cells, one mass with vestigial collars, remainder without collars. Flagellar action moderate, one with shortened flagella. Blow-outs shrunken in all but one. Some cell-disintegration.

2 hrs. No collars. Flagellar action slow and spasmodic.

18 hrs. No flagella. All masses with much disintegration into separate cells.

D. $\frac{n}{200,000}\ HgCl_2$.

1 hr. No collars. Flagellar action very slow or nil. Flagella absent or normal length. Two blow-outs still present.

1 hr. 55 m. No flagella. Disintegration starting in all.

18 hrs. 45 m. Masses present, but more disintegrated than C.

E. $\frac{n}{100,000}$ $HgCl_2$.

50 m. Collar-cell blow-outs no longer visible. One dermal blow-out unaffected. Two masses with a few flagella moving (slow or spasmodic). Flagella somewhat shortened. Masses with irregular outlines.

1 hr. 50 m. All cells rounded off, total disintegration of masses starting.

18 hrs. 30 m. Completely disintegrated into groups of one to twenty dead cells.

F. $\frac{n}{50,000}$ $HgCl_2$.

40 m. No collars or flagella. Blow-outs burst, contracted, or disappeared. Masses with irregular outlines.

1 hr. 50 m. and 18 hrs. 30 m. As E.

10. Discussion.

(*a*) Dedifferentiation. Position and Fate.

Wilson, in his work on dissociation and subsequent regeneration in Monaxonid sponges, left the question entirely open as to whether regeneration was due wholly to the 'totipotent' amoebocytes, or whether the differentiated tissue elements underwent a process of 'despecialization' (dedifferentiation) into an 'indifferent or totipotent state', after which they took their shares in restitution. He does not seem to have envisaged the possibility of the differentiated cells sharing in the restitution-process without undergoing total dedifferentiation. In his later paper, on dissociation and restitution in Hydroids (**16**), he returns to the subject, and decides that in these forms, where undifferentiated cells form but a fraction of the normal body, the differentiated tissue-elements definitively become despecialized 'to form masses of totipotent regenerative tissue'. The cells in these masses later differentiate in accordance with their position, the outer cells forming ectoderm, the central core endoderm. This is, of course, in accordance with Driesch's well-known dictum that the fate

of a cell is a function of its position. He finally concludes that, since the restitution-masses of sponges are so like those of Hydroids, the processes occurring in them are of the same nature.

He further stated that the cells in the early stages of the restitution-mass formed a syncytium, few or no cell-boundaries being distinguishable.

De Morgan and Drew, in their later work on restitution-masses in other Hydroids, while confirming Wilson in some points, differ from him in others. In the first place, although restitution-bodies with perisarc, ectoderm, and typical endodermic coenosarcal tubules were produced and lived for as long as sixty days, no hydranths were formed. As Orton (**12**) suggests, this may be due to the fact that de Morgan and Drew's experiments were performed from December to March, when the growth of Hydroids appears to be at a standstill, while Wilson worked in the summer.

In the second place, although they describe a syncytial phase, their figures do not show any such complete cell-fusion as Wilson's, and they mention that a small portion of endoderm cells are always to be recognized as such. They do not pronounce definitively one way or the other as to whether dedifferentiation of all cells to a ' totipotent ' condition occurs.

Müller (**10**) also believes that collar-cells do not take part in the redifferentiation of restitution-masses in Spongilla, but that the amoebocytes and thesocytes form the new gastral cells. In view of the great rôle played by the amoebocytes in monaxonid sponges, and the specialization, small size, and relatively small number of the choanocytes, this is not surprising. In gemmule development, for instance, the flagellated chambers arise from archaeocytes. The same author (**11**), in describing dedifferentiation in Spongillidae, notes that the choanocytes early dedifferentiate and disappear, apparently ingested by amoebocytes. It would appear that they cannot maintain themselves as such in unfavourable conditions. In this connexion, mention may be made of the work of Maas (**9**), who found that slow deprivation of calcium led to similar

dedifferentiation in various calcareous sponges, including a heterocoelous form (Sycandra). He also describes degeneration and phagocytosis of the collar-cells in late stages of the process.

In view of my work (see discussion below), it would appear that in both Calcarea and Monaxonida the choanocytes are more susceptible than the amoebocytes, and will degenerate in certain conditions. In Calcarea, however, this difference in susceptibility is less marked, and the choanocytes will remain capable of maintaining existence in dissociation-masses, while this is not possible for those of Spongilla.

My own work (7) on Sycon indicated that the conclusions of Wilson as to the fate of the cells in restitution do not apply in the case of Sycon. On dissociation the tissue elements all become dedifferentiated morphologically, e. g. the choanocytes lose both collar and flagellum and become rounded, the dermal cells lose their extended flat shape for a spheroidal one ; but this dedifferentiation is not complete in the sense that the various kinds of cells become physiologically similar, or acquire the same potentialities of development. After this dedifferentiation caused by shock the cells redifferentiate in appropriate directions, the dermal cells producing an external epithelium round a central choanocyte mass, which in its turn becomes hollow with epithelial walls. The normal form of the post-larval sponge is thus produced by a process exactly the reverse of that envisaged by Driesch and Wilson. The fate of the cells is not a function of their position, but their eventual position is a function of their constitutional differences. The development of a restitution-body is primarily a process of sorting-out of different kinds of cells, followed by a redifferentiation of the individual types of cells. We have thus to distinguish sharply between two types of cellular dedifferentiation : (1) that which leads to complete loss of the character of the tissue to which the cell belongs, and a return to a totipotent, or at least, if I may coin a new word, to a pluripotent condition. This may be called ultra-typical (or pluripotent) dedifferentiation; (2) that which leads to a temporary

suppression of the characters of the cell, also with the assumption of a simple spheroidal form. Redifferentiation, however, is only possible in the direction of the original form, and the cell has not acquired pluripotency by dedifferentiating. This may be called intra-typical (or unipotent) dedifferentiation.[1]

The existence of pluripotent dedifferentiation is rendered probable by various observations which cannot be entered into here. It has frequently been assumed, however, on insufficient evidence; and in view of its theoretical importance, and the difficulty of proof, very thorough investigation is required to establish its existence in any particular case.

Further evidence against its occurring in Sycon was afforded by the artificial production of masses composed entirely, or almost entirely, of collar-cells. These, though they lived healthily for a number of weeks, never produced a dermal epithelium or spicules. This is paralleled by the failure of endoderm or ectoderm alone to regenerate in Hydra, as has been shown by various observers.

In a later paper (Huxley, **8**) attention was drawn to the fact that masses composed only of collar-cells were less viable than those containing dermal cells also, although both were kept under identical conditions, and although the collar-cells are the organs of nutrition.

In the present paper, the converse of the collar-cell blow-outs is shown to occur in the form of masses with an excess of dermal cells. These form blown-out vesicles exactly as do the choanocytes when they are in excess.

It is thus clear that, in Sycon at least, the form and composition of the restitution-mass depends (apart from questions of size) upon the proportions of the different types of cells which entered into its composition.

It is clear from the observations of Wilson that some process of dedifferentiation does occur in restitution-masses of Hydroids.

[1] Since writing the above, I find that a very similar classification of types of dedifferentiation from the point of view of tumour-growth has been adopted by Adami and McCrae (**1**, p. 324. See also pp. 318-22).

E.g. in Pennaria the endoderm cells enter in large numbers into the composition of the restitution-masses, and are distinguishable immediately after dissociation by large granules. Within twenty minutes, however, a syncytial mass has been formed, in which very few of these granular elements can be distinguished. Presumably the granules have been resorbed in the new conditions. On the other hand, neither his observations, nor those of de Morgan and Drew, in the least exclude the idea of migration of ectoderm or endoderm cells to their proper stations after intra-typical dedifferentiation.

In this connexion, the facts concerning the possible attraction of the various types of cells for each other may be mentioned (Huxley, 8). In cultures consisting almost entirely of collar-cells, a small proportion of normal regenerates usually occurred. In other cultures made at Plymouth, where the great majority of the masses were choanocyte blow-outs, with partial dermal covering, a small proportion were dermal blow-outs. These facts may be due either to accidental distribution of dermal cells, or else to an attraction of dermal cells for each other. This point could only be settled by appropriate experiments. The probable attraction of spermatozoa by choanocytes was mentioned in the same paper.

(*b*) Formation of Blow-outs.

The secretion of fluid by epithelia, whether dermal or choanocyte, and consequent formation of spheres or segments of spheres (' blow-outs '), is an interesting phenomenon.

In this connexion, Mr. J. Gray, of King's College, Cambridge, has kindly allowed me to refer to some unpublished observations of his own, which he is at present investigating, on the formation of similar blown-out spheroidal masses by fragments of the gills of Mytilus. The phenomenon would thus seem to have more than isolated significance. It perhaps involves changes of the same nature as those taking place in the formation of a blastocoele.

(c) Size Relations; Viability.

Wilson (loc. cit.) found that the size of the restitution-masses produced by Hydroids was of great importance. Large masses almost invariably died early, while too small masses, though living for a long time, failed to produce Hydranths or even coenosarcal outgrowths.

In Sycon also, very small masses, though reaching a two-layered stage and occasionally forming spicules, fail to metamorphose. Similar failure to produce normal structure from pieces below a certain definite size is well known in studies on regeneration, both in unicellular and multicellular organisms. It may be partly due to mere lack of material, but undoubtedly also, in some way not as yet properly understood, to the relatively greater surface and the consequences thereon attendant—differences of gaseous exchange and difference of stimulation by the environment being prominent.

Similarly, in too large a mass, it does not appear that proper oxygenation for the central cells can be provided, and so disintegration sets in. Wilson found the interesting fact that successfully-metamorphosing masses were of the same order of size as normal planulae. The same is roughly true for Sycon, although here the upper limit of size for successful masses is much further above the larval size than in Hydroids.

De Morgan and Drew comment on the fact that their restitution-masses, although not metamorphosing, were much more resistant to laboratory conditions than the normal colonies, and regard it as surprising. There should be no ground for surprise in this—the cells of the restitution-masses are definitively, as we have seen, in a dedifferentiated condition. Experiments on Perophora and Obelia show that the undifferentiated stolons and hydrocaulus remain perfectly healthy in conditions causing dedifferentiation and resorption of the zooids. Clavellina and other Ascidians hibernate in the form of 'winter-buds', which are of somewhat similar nature to restitution-masses; and the normal gemmules of sponges have also something in common with them. In the laboratory the hydriform

larva of the medusa Gonionema becomes transformed into a syncytial, undifferentiated mass, as was shown by Perkins (**13**).

The obverse of this condition is shown by the failure of highly differentiated parts of the organism to maintain themselves in the restitution-masses. Wilson and de Morgan and Drew found that portions of tentacles fail to become incorporated in the masses. This is paralleled by the failure of Hydra tentacles to regenerate. Apparently, on the one hand they are too highly specialized to dedifferentiate ; and on the other cannot exist as such in the conditions afforded by the restitution-bodies. The nematocysts also are gradually resorbed in the restitution-bodies.

If we seek to embrace the phenomena in one general view, we may say that Hydroid tissues in unfavourable or abnormal conditions lose much of their differentiation, come to have a low metabolic rate (in the general sense in which the term is used by Child (**3**)), and are more resistant. In these conditions specialized organs cannot exist. The same tissues in optimum conditions possess a higher metabolic rate, and are capable of maintaining specialized organs such as the tentacles in existence.

(*d*) 'Normal' and 'Abnormal' Phenomena.

Attention has already been drawn to the fact that many of the processes occurring in restitution-bodies and free tissue-cultures run parallel with various phenomena of development. The normal phenomena constitute an interlocking series, each stage of which is determined by the preceding and helps to determine that which comes after. By studying processes which occur in 'abnormal' conditions, e.g. by dissociation methods, we remove the tissues of the organism from this developmental chain, where it is often impossible to say what occurrences are palingenetic, what adaptive, what the direct consequence of changes in the environment, and what conditioned by previous processes in the series ; by varying the conditions, we may then throw light upon the normal processes.

So far my work has been mainly devoted to elucidating

roughly the course of events in restitution-bodies in Sponges. It is clear, however, that in Sycon we have an admirable material for qualitative experiment, as to the rôle of size of masses, the proportion of the tissues in the mass, the coherence of cells, their mutual attraction, &c.

The elucidation of these problems will need many workers, and it is hoped that others may be induced by the facts here set forth to take up the work.

Meanwhile two tendencies should be noticed. The first is a tendency to discuss the results from a morphological standpoint. This is shown, e.g., in Wilson's discussion of results. He compares the development of the restitution-masses in detail with that of normal development, and goes so far as to apply the term 'yolk' to the central syncytial portion which remains in the middle of the masses while the two layers are differentiating. This, and indeed his whole discussion, though of great interest, seems to me to be putting the cart before the horse. We should rather expect to find some of the causes determining the presence and form of the normal yolk by examining the mode in which the abnormal conditions of a restitution-mass influence the internal cells, rather than vice versa.

A word is also in order as to the use of the terms 'normal' and 'abnormal'. Abnormal is often used as if it were synonymous with pathological. This is not the case in any of the forms of restitution-mass here described (until we reach degenerative change at the close of their history, this being due to lack of nutriment and to laboratory conditions). Dedifferentiation, aggregation, sorting-out, &c., are all perfectly healthy phenomena.

11. Summary of Results.

(Including those recorded in previous papers.)

1. Various methods can be used to dissociate the tissues of *Calcarea Heterocoela*.

2. Mixture of the various types of cells in normal proportions may lead to the formation of normal regenerates, resembling post-larval Sycon, with spicules, osculum, and pores.

3. The development of these masses consists primarily in the sorting-out of the dermal and gastral cells. The former produce a single-layered epithelium, below which spicules are subsequently formed, the latter a central mass which later becomes a hollow one-layered sac, into whose cavity the cells put forth collar and flagella. Thus their fate is not a function of their position in the whole, but their position a function of their nature.

4. The two types of spicules are formed in the same order as in normal development.

5. Free tissue-cultures consisting only of collar-cells can be obtained by appropriate methods. These form spheres resembling choanoflagellate colonies with the collars directed outwards. These live for a considerable time, but do not regenerate other forms of tissue or produce spicules.

6. All grades from these to masses containing an excess of dermal cells may be formed. They may be classified as follows :

(*a*) Collar-cell spheres.

(*b*) Collar-cell blow-outs. These consist of a solid mass with one or more portions blown out to form a segment of a collar-cell sphere.

(*b* 1) With active collar-cell epithelium over the whole surface.

(*b* 2) With mixed collar-cell and dermal epithelium over the solid portion.

(*b* 3) With dermal epithelium over the solid portion.

(*c*) Normal regenerates.

(*d*) Dermal blow-outs, resembling (*b* 1), but with dermal epithelium over the whole surface.

7. In almost pure collar-cell cultures, a few normal regenerates may be found. In cultures consisting almost entirely of collar-cell blow-outs, a few dermal blow-outs may be found. This is probably due to mutual attraction of dermal cells.

8. Normal regenerates are more viable than collar-cell spheres or collar-cell blow-outs of type (*b* 1).

9. Dermal blow-outs may be formed from collar-cell blow-outs. They are in such cases produced more readily from large masses.

10. Numerous methods have been tried for feeding the collar-cell spheres and blow-outs, but so far without success.

11. The flagella of collar-cells are adhesive.

12. Larvae may become embedded in the restitution-masses ; they are gradually resorbed.

13. Restitution-masses, if brought into contact, will cohere. The irregular masses thus produced gradually round up and become unified.

14. Mechanical shock causes a contraction of both dermal and choanocyte blow-outs, and a retraction of the collars and partial retraction of the flagella in the latter.

15. A peculiar small finger-shaped amoebocyte (' finger-cell ') is numerous in normal sponges and restitution-masses. These cells are arranged in a remarkable manner below the dermal epithelium of dermal blow-outs.

16. Spontaneous segmentation of restitution-masses into small spherules may take place, apparently in unfavourable circumstances. The spherules usually secrete a gelatinous covering. They may differentiate a normal dermal epithelium. The bulk of the component tissue (presumably choanocyte) usually separates into its constituent cells after a time.

17. A type of restitution-body with dark central mass is described.

18. Dedifferentiation of all cells takes place after dissociation, but does not lead to a totipotent condition.

New College, Oxford.
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EXPLANATION OF PLATES 13 AND 14.

The figures are all drawn from life with the Abbé camera lucida. The magnifications are given as follows: 3+4 oc., denotes drawn at table level with a no. 3 ($\frac{1}{3}''$) objective and no. 4 Huyghenian ocular. The objectives and oculars were Reichert unless otherwise stated.

Plate 13.

Fig. 1.—A subdivided restitution-mass. (Eight days.) *a*. The whole mass. The spherules are mutually compressed and show a definite cubical epithelium. (3+4 oc.) *b*. A single spherule under higher power. The central mass is distinct from the epithelium. (6+2 oc.)

Fig. 2.—Different stages of another subdivided mass. (3+4 oc.) *a*. A nine-day mass. The spherules have separate gelatinous layers, and no sharp epithelia. Dark areas are seen within them. *b*. Three days later. All but one possess well-marked dermal epithelia and have somewhat expanded. The central masses are irregular, and several have fragmented.

Fig. 3.—A small eleven-day mass with dermal epithelium; the contents are subdivided into small spherules. No jelly-layer. (3+4 oc.)

Fig. 4.—Ten-day subdivided masses. The individual jelly-layers of the spherules are not shown. (3+4 oc.)

Fig. 5.—A single spherule of the mass of fig. 4, three days later, under higher magnification. The layer of jelly, the separation of the clear cells, and

the dense mass of yellow-brown cells are seen. (Zeiss $\frac{1}{16}''$ water-immersion + 4 compens. oc.)

Fig. 6.—Another spherule from the same specimen, same date. The layer of jelly is thinner. The spherule has subdivided into irregular masses with clear outer layer and yellow inner centre. From one, cells are beginning to separate. (Same magnification as 5.)

Fig. 7.—Olynthus stages from restitution-masses. Osculum and oscular crown are well developed. (3 + 2 oc.) *a*. Large, fixed, of normal shape (spicules figured at the edge only). *b*. Smaller, of abnormal shape (spicules omitted). A small patch (undotted in the figure) lacks the gastral layer.

Plate 14.

Fig. 8.—A further stage in the development of the type shown in fig. 13. The gastral layer is markedly incomplete (spicules only figured at the edge). (3 + 4 oc.)

Fig. 9.—Successive stage in one hanging-drop culture. (3 + 4 oc.) *a*. The chief masses present in the drop, two hours after isolation (four days from beginning of experiment). *b*. After two days. The masses shown in (*a*) have fused together (in addition, in (*a*) there were three embryos and two small masses which had not fused). Note three larvae and one sphere partially attached. *c*. After four days. Larvae no longer visible, blow-out larger; more unification of the separate masses. *d*. After six days. No collars. Flagella shorter and fewer. Still more unification. Gaps in the blown-out region bridged by dermal membranes with amœbocytes on the inner surface. *e*. After nine days. Disappearance of blow-out. No collars or flagella. *f*. After thirteen days. Still further contraction. A few cells had separated from the mass (not shown).

Fig. 10.—To show the effects of mechanical shock. (3 + 4 oc.) *a*. A mass with good choanocyte blow-out and attached larva. *b*. The same mass after repeated pipetting. The larva is detached, the epithelium of the blow-out has contracted and thickened, the collars have been retracted.

Fig. 11.—Restitution-mass with dermal epithelium and central dark yellow-brown sphere, separated from intermediate layers of collar-cells. (6 + 2 oc.)

Fig. 12.—Small dermal blow-out under high power. (6 + 2 oc.) The dermal cells are granular. Adhering to the inner side of each are a number of finger-cells. A few dermal cells are figured in surface view. From others, the subjacent finger-cells have been omitted. Over the rest of the surface, dermal cells are not figured. The bulk of the interior mass is composed of choanocytes. From the edge of this, finger-cells protrude into the blow-out cavity.

Fig. 13.—Very large dermal blow-out, spherical type. (3 + 4 oc.) Here there is no sharp internal mass, but the collar-cells form irregular areas of varying thickness adherent to the dermal epith[illegible]. Those on the upper side are represented darker than those below. [illegible]ne cells of the dermal epithelium have been represented too large.)